

Detection of Exotoxins and Antimicrobial Susceptibility Pattern in Clinical *Pseudomonas Aeruginosa* Isolates

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Abstract

Background: *Pseudomonas aeruginosa* is a common opportunistic pathogen that causes nosocomial infection in immunocompromised patients. Among different virulence factors, the type III secretion system (TTSS) is an important agent in virulence and development of antimicrobial resistance in *P. aeruginosa*.

Objectives: Previous studies have shown that production of type III secretion system proteins was correlated with increasing virulence and resistance to several antibiotics. In this study we determined the exotoxins genes (*exoU* and *exoS*) and pattern of antimicrobial susceptibility in clinical *P. aeruginosa* isolates.

Methods: A total of 175 *P. aeruginosa* isolates were collected from patients hospitalized in educational hospitals of Shahrekord and Chamran hospital of Isfahan, Iran from April to December 2015. Antimicrobial susceptibility test was performed by disk diffusion test. The presence of exotoxins genes was detected using multiplex PCR of *exoU* and *exoS* genes.

Results: The antibiotic resistance rate was higher than 70% to many antibiotics. The highest rate of resistance was related to Levofloxacin and Meropenem (155 (88.6%), 148 (84.6%)) respectively. The *exoU* gene was found in 75 (42.9%) isolates and 136 (77.7%) of the isolates carried the *exoS*. In addition, 36(20.6%) of the isolates carried the both of gens. A statistical significance was detected between the presence of *exoU* gene and resistance to piperacillin (P = 0.01).

Conclusions: The result of this study was indicated a high resistance rate to the most antibiotic classes and a specific relationship between the virulence genotype and antimicrobial resistance especially more virulent genotype of *exoU*⁺. In order to prevent the spread of more virulent strains in health care facilities, molecular methods alongside antimicrobial susceptibility tests is suggested.

Keywords: Virulence Factors, Genotype, Type III Secretion Systems, *Pseudomonas Aeruginosa*

1. Background

Pseudomonas aeruginosa is a Gram-negative and an opportunistic pathogen which grow in minimal nutritional requirements and a wide range of temperature. It can grow on most of surfaces, especially moist surfaces such as medical devices and skin (1, 2). *Pseudomonas aeruginosa* in weakened and immunosuppressed patients, who are with third-degree burns, cystic fibrosis (CF), wounds, indwelling catheter and prolonged duration of ventilation it can cause nosocomial diseases (3). Multiple factors are involved in pathogenicity of *P. aeruginosa*. The type III secretion system (TTSS) has been known to be a major virulence, which is determined in pathogenesis of acute infection, bacteremia, sepsis and subsequent mortality. The TTSS allows the injection of toxins into the cytosol of target eukaryotic cells, where they destroy host cell defense and

signaling systems and that subsequently rapid call necrosis or modulating the actin cytoskeleton (4-6). Four effector proteins have been identified: ExoU, which is a phospholipase and it has been characterized as a major virulence factor in acute lung injury, ExoY that is an adenylate cyclase, and ExoS and ExoT which are bifunctional proteins (5, 6). ExoU and ExoS are present variably and in pathogenesis are important, whereas the almost of isolates, ExoT and ExoY encode and have a minor effect on virulence (4, 5). Previous studies have shown that production of ExoU was correlated with increasing virulence (7). Other studies were found that the infected patients with TTSS⁺ isolates show more severe infections and the mortality rate of this patients in first 30 days of infection is high (3, 8). The existence many of agents in *P. aeruginosa* leads to intrinsic resistance to many antimicrobials including bacterium's outer membrane barrier, the presence of multi drug efflux

trans-porters and endogenous antimicrobial inactivation. The all of this agents, also inappropriate chemotherapy and lagging in antibiotic discovery was caused “antibiotic resistance crisis” (6, 9). The previous studies were showed that the inappropriate chemotherapy leads to emerge of the multi-drug resistant isolates (10), especially in burn patients, the rate of resistance to most of antibiotics for *P. aeruginosa* isolated was reported higher than 70% (11). However, the ExoU⁺ isolates are more resistance to fluoroquinolones (12).

2. Objectives

The aim of this study was identification of antimicrobial susceptibility pattern and characterization the presence of *exoS* and *exoU* genes in clinically isolated *P. aeruginosa* strains. An improved understanding of these virulence factors is important for selection cure pathway and suitable antibiotic.

3. Methods

3.1. Isolation and Identification of Bacteria

In a descriptive study which is approved by the ethics committee of Shahrekord University of Medical sciences (research project number 2450), a total of 175 non-replicated *P. aeruginosa* isolates were collected from April to December 2015 at teaching hospitals of Shahrekord University of Medical Sciences and Chamran hospital of Isfahan, Iran. These isolates derived from routine diagnostic tasks and different specimens including wound, blood, urine, specimens respiratory, burn wound, (cerebrospinal fluid) CSF and others. After transporting the isolates to Shahrekord University of Medical microbiology laboratory, the isolates were identified as *P. aeruginosa* by conventional methods (Gram staining, catalase, oxidase, non-fermentation in (oxidative fermentation) OF culture and production of pigmentation) (13).

3.2. Antibiotic Susceptibility Test

Antibiotic susceptibility testing of the isolates was performed using Kirby-Bauer disk diffusion method according to clinical and laboratory standard institute guideline (CLSI, 2015) (14). The tested antimicrobial agents were as followed: Imipenem (10 µg), Meropenem (10 µg), Doripenem (10 µg), Levofloxacin (5 µg), Ciprofloxacin (5 µg), Ceftazidime (30 µg), Cefepime (30 µg), Colistin (10 µg), Polymyxin B (30 µg), Gentamicin (10 µg), Amikacin (30 µg), Tobramycin (10 µg), Piperacillin (100 µg), Piperacillin/Tazobactam (100/10 µg), and Aztreonam (30 µg) (MAST, Group Ltd, Merseyside, UK). *Escherichia coli*:

ATCC25922 and *P. aeruginosa*: ATCC27853 were used as quality control (CLSI, 2015).

3.3. DNA Extraction

DNA extraction was performed by boiling method with some modification. An aliquot of 1 mL of the culture grown overnight at 37°C in 100 µl of (Tris EDTA Acid Acetic)TEA buffer was boiled for 10 minutes at 100°C and immediately placed in -20 °C for 10 minutes. After centrifugation of bacterial suspensions at 13000 × g at 4°C for 10 minutes, the supernatant was collected and DNA template used for polymerase chain reaction (PCR) (15).

3.4. Virulence Genotyping Test Using Multiplex PCR

For identification of *exoU* and *exoS* genes in *P. aeruginosa* isolates multiplex polymerase chain reaction (MPCR) were performed. Two set of primers were used that are shown in Table 1 (synthesized by Bioneer, Inc. Seoul, South Korea). MPCR mixture consisted of 1X reaction buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0)), 2 mM MgCl₂, 200 µM concentration of each of four deoxyribonucleoside triphosphates (dNTPs) (Sina Clon Bio Science Co. Tehran, Iran), 0.4 µM primers, 5 U of Taq DNA-polymerase (Sina Clon Bio Science Co. Tehran, Iran) and 50 ng DNA. Amplification was performed in a T100™ Thermal Cycler (Bio-Rad Laboratory, Inc. USA) (16). After an initial denaturation step for 5 minutes at 94°C, 30 cycles of amplification were performed as follows: 94°C for 30 seconds, 59°C for 45 seconds, and 72°C for 45 seconds. The reaction was completed with a final extension at 72°C for 10 minutes. The Amplified products were analyzed by 1.5% agarose gel electrophoresis which is visualized on an ultraviolet illumination.

Statistical package for the social sciences version 22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Fisher's exact test or Chi-squared test was performed in order to analyze of categorical data. A P value of < 0.05 was considered statistically significant.

4. Results

The results of antimicrobial susceptibility testing are shown in Table 2. All of the isolates were susceptible to colistin and polymyxin B and the most of them was resistant to Levofloxacin (155 (88.6%)) and Meropenem (148 (84.6%)). The highest susceptibility was related to piperacillin/Tazobactam (59 (33.7%)) and Amikacin (54 (30.9%)); respectively. Multiplex PCR in order to detect of *exoS* and *exoU* genes was performed for all isolates. Gel electrophoresis results of a number of isolates are shown in Figure 1. All 175 clinical isolates carried *exoS*, *exoU* and *exoU/exoS*. The *exoU* gene was found in 75 (42.9%) isolates

Table 1. Oligonucleotide Primers Used in This Study

Name	Primer Sequence	References	Size of the Amplicon, bp
ExoU-F ^a	5'-AGC GTT AGC GAC GTG CGT TTC A-3'	This study	404 ^c
ExoU-R ^b	5'-GCG CCG ATC TCG CTG CTA ATG T-3'		
ExoS-F	5'-TTC GGG CAG GGC ACG ATA TCC A-3'	This study	240
ExoS-R	5'-TTC CGG TTT GCT TGC CAG GTC G-3'		

^aForward primer.^cBase pair.^bReverse primer.

and 136 (77.7%) of isolates had *exoS* gene. Furthermore, 36 (20.6%) of the isolates carried the both of genes. Statistical analysis has been showed a significant difference between the presence of *exoU* gene and resistance to piperacillin (P = 0.01). However, there was not detected significant difference between the presence of exotoxin genes and other antibiotic resistance. Frequency of *exoU*⁺ and *exoU*⁻ among piperacillin-resistant and non-resistant isolates was shown in Figure 2.

Table 2. The Results of Antimicrobial Susceptibility Testing in *P. Aeruginosa* Clinical Isolates^a

Antibiotics	Sensitive	Intermediate	Resistance
Imipenem, 10 µg	25 (14.3)	7 (4.0)	143 (81.7)
Meropenem, 10 µg	21 (12.0)	6 (3.4)	148 (84.6)
Doripenem, 10 µg	29 (16.6)	10 (5.7)	136 (77.7)
Levofloxacin, 5 µg	16 (9.1)	4 (2.3)	155 (88.6)
Ciprofloxacin, 5 µg	26 (14.9)	6 (3.4)	143 (81.7)
Ceftazidime, 30 µg	35 (20.0)	1 (0.6)	139 (79.4)
Cefepime, 30 µg	27 (15.4)	9 (5.1)	139 (79.4)
Colistin, 10 µg	175 (100)	0 (0.0)	0 (0.0)
Polymyxin B, 30 µg	175 (100)	0 (0.0)	0 (0.0)
Gentamicin, 10 µg	30 (17.2)	6 (3.4)	139 (79.4)
Amikacin, 30 µg	54 (30.9)	13 (7.4)	108 (61.7)
Tobramycin, 10 µg	33 (18.9)	1 (0.6)	141 (80.6)
Piperacillin, 100 µg	29 (16.6)	51 (29.1)	95 (54.3)
Piperacillin/tazobactam, 100/10 µg	59 (33.7)	46 (26.3)	70 (40.0)
Aztreonam, 30 µg	28 (16.0)	18 (10.3)	129 (73.7)

^aValues are expressed as No. (%). No, number of strains.

5. Discussion

Among the all pathogenic bacteria, *P. aeruginosa* contributes to 11% of all nosocomial infections (17). The clinical

P. aeruginosa isolates that secret type III proteins (ExoU and ExoS) show more antimicrobial resistance and associate with increase rates of mortality and morbidity (3).

In this study, all of the isolates showed at least one of the exotoxin and the prevalence of *exoU*⁺/*exoS*⁺, *exoU*⁻/*exoS*⁺ and *exoU*⁺/*exoS*⁻ clinical isolates were totally 42.9%, 77.7% and 20.6%; respectively. In the several studies that have been conducted on *P. aeruginosa* isolates, the presence of exotoxin gene has been reported differently. In a study by Cho et al., 66 carbapenem-resistant *P. aeruginosa* isolates from different clinical source were investigated and the prevalence of *exoU*, *exoS* and *exoU*/*exoS* genes were reported 66.7%, 30.3% and 3%; respectively (5). These findings are contrary to our results. In a study which is conducted by Ferreira et al., all of 32 *P. aeruginosa* isolates from patients with bacteremia and ventilator associated pneumonia (VAP) carried *exoS* gene. The *exoU* gene was observed only in 9.4% of strains and three isolates were positive for the two effector genes *exoU* and *exoS* (9.4 %) (10). In another study by Mitove and colleagues on *P. aeruginosa* isolated from patients with CF and nosocomial infections, the prevalence of *exoS* and *exoU* have been reported 62.4 and 30.2%; respectively (18). Similar to our results, in this study the prevalence of *exoU*⁺/*exoS*⁺ isolates was more than the *exoU*⁺/*exoS*⁻ isolates. However, in our study the prevalence of *exoU*⁺/*exoS*⁻ was higher than those. In the study by Firuzi-Dalvand et al., the prevalence of *exoU* and *exoS* genes in burn wound *P. aeruginosa* isolates were reported 76% and 68%; respectively (19). In another study that was conducted by Agnello et al., the prevalence of *exoU*, *exoS*, *exoU*/*exoS* or other exotoxin genes were 38%, 56% and 8%; respectively (4). Jabalameli et al., were reported the frequency of *exoS*, *exoU* and *exoU*/*exoS* in burn isolates 29%, 64.5% and 1%; respectively (20), which their finds was conducted with ours. In a survey that conducted by Lakshmi Priya et al., all of the isolates were carried one of the *exoU*, *exoS* or *exoU*/*exoS* genes. The frequency of *exoS* and *exoU* genes has been detected 64% and 23%; respectively (21). Garey et al. in a survey on *P. aeruginosa* isolates from

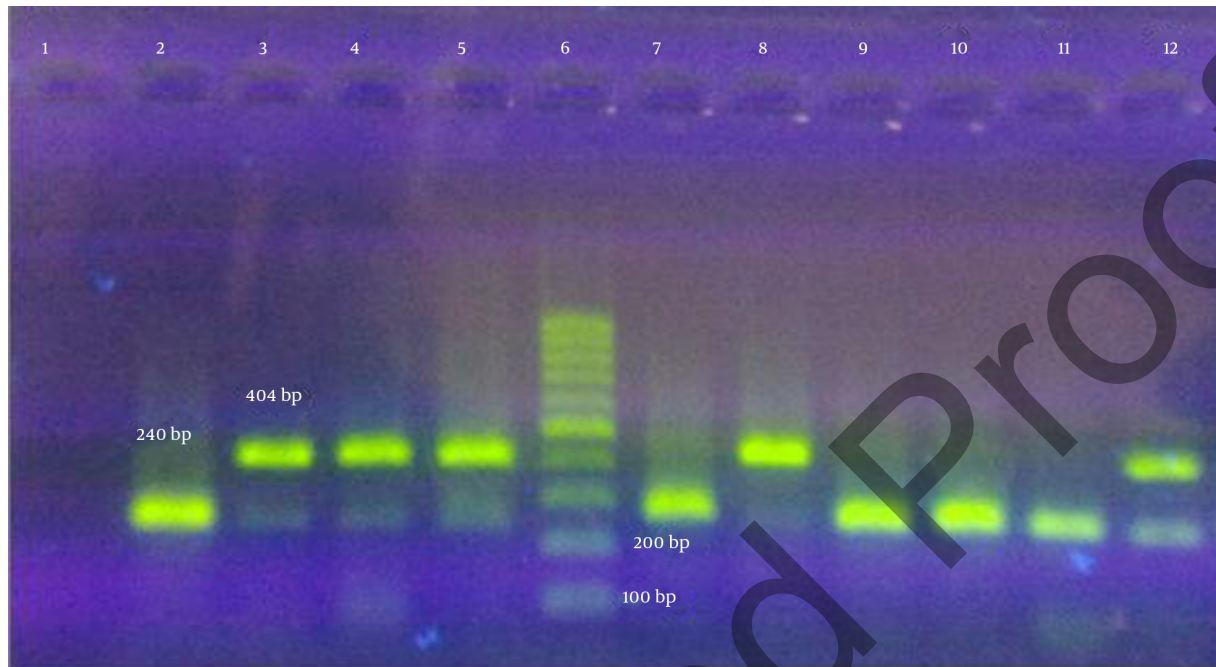


Figure 1. Gel Electrophoresis Results of *exoU* Gene (404 bp) and *exoS* Gene (240 bp). Lane 1: Negative Control, Lane 6: 100 bp DNA Ladder, Lanes 2, 7, 9, 10 and 11: Isolates with *exoS* Gene, Lanes 3, 4, 5 and 8: Isolates with *exoU* Gene and Lane 12: Positive Control

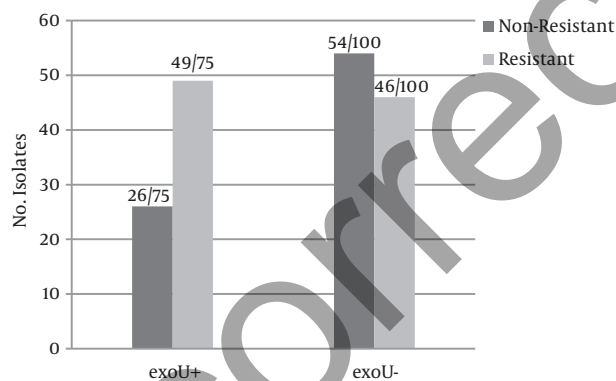


Figure 2. Frequency of *exoU*⁺ and *exoU*⁻ among piperacillin-resistant and Non-resistant isolates

patients with bacteremia reported that the prevalence of *exoS*, *exoU* and *exoU/exoS* were 70.5%, 24.5% and 1.6%; respectively (22). Wong-Beringer et al. was found that the prevalence of *exoS* and *exoU* genes in clinical isolates were 62% and 27% respectively. These findings were near to our results (12). According to the mentioned studies and compared them with our study, the observed differences in the prevalence of *exoS* and *exoU* genes may be due to the diversity in the type of specimen, strain types and geograph-

ical area of survey. Notably, in our study the percent of *exoU*⁺/*exoS*⁺ isolates were relatively high (20.6%) compared to other studies. Even though *exoS* and *exoU* genes are located in different location in *P. aeruginosa* genome, the simultaneous carriage of both genes less have been reported (23). A possible explanation is that both of the *ExoU* and *ExoS* proteins are important for *P. aeruginosa* survival. However each of them shows different activity in a specific environment and situation (23, 24). Therefore, the exotoxins genotype of a clinical isolate may be indicated of particular environmental reservoir of that isolate.

Many cases of infections due to *P. aeruginosa* were hardly cured and also inappropriate chemotherapy was caused resistance to many of antibiotics (24, 25). The extensive use of antibiotics such as flouroquinolones can be effective in creating the multi-drug resistant isolates. In addition, previous studies were shown that the presence of the TTSS effector genotypes, especially *exoU* genotype, correlate with increasing the virulence and resistance to one or more antibiotics. In a study by Agnello et al., they were found that the presence of *exoU* gene was significantly correlated with flouroquinolones- resistance (4). Wong-Beringer et al., also found that 92% of *exoU*⁺ and 61% of *exoS*⁺ isolates were resistance to flouroquinolones (12). In other study by Cho et al., a higher ratio of *exoU*⁺ strains were flouroquinolones-resistant than *exoS*⁺ strains ($P \leq$

0.0001) (5). In surveys that conducted by Maatallah and et al., and Pena and et al., the presence of *exoU* gene significantly was correlated with multidrug resistance and ciprofloxacin resistance (23, 26). In present study 81.7% and 88.6% of isolates were resistant to ciprofloxacin and levofloxacin; respectively. On the contrary to other studies, there was no correlation between the resistance to fluoroquinolones and specific virulence genotype. Although, resistance to Piperacillin significantly was associated with presence of *exoU* gene ($P = 0.01$). One reason for this difference may be due to a variation of sample type but further studies should be conducted in order to find out this difference.

Our study has some limitation such as single center study with small sample and lack of funds for more experiments such as Clonality analysis. However, since the study on distribution of TTSS effector genotypes and antibiotic resistance in our region is very low, further studies, like our study, are required.

5.1. Conclusion

In use together, the recent overuse of effective antipseudomonal antibiotics has led to increased resistance in clinical *P. aeruginosa* isolated. The present results indicate a specific relationship among the presence exotoxin genes and antibiotic resistance. In order to prevent the spread of more virulent strains in health care facilities, molecular assessment alongside antimicrobial susceptibility tests is suggested.

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Footnotes

Authors' Contribution: Somayeh Malek Mohamad contributed to sample collection and experiments; Soodabeh Rostami contributed to study design; analyzed the data: Fathemeh Drees; wrote the paper: Somayeh Malek Mohamad and Soodabeh Rostami.

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